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Calof AL, et al

Regulation of neurogenesis and neuronal differentiation in primary and immortalized cells from mouse olfactory epithelium.  
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# Regulation of neurogenesis and neuronal differentiation in primary and immortalized cells from mouse olfactory epithelium

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**Abstract.** We have developed an *in vitro* system for studying molecular events regulating neurogenesis in the mouse olfactory epithelium (OE). Our observations suggest that two types of neuronal precursor may be involved: (1) a transiently existing, immediate neuronal precursor (INP), which generates two postmitotic daughter neurons; and (2) a neuroepithelial stem cell, which may be the basal cell (or some subclass of basal cell) of the OE, and is presumed to be the progenitor of the INP. Using antibody markers that distinguish basal cells and postmitotic receptor neurons *in vitro* and *in vivo*, we have shown that neurogenesis occurs early on in OE cultures, but then ceases because INPs divide only once to generate postmitotic neurons and no new INPs are produced by basal cells. To determine whether the basal cell-to-INP transition, or proliferation and neuronal differentiation of the INP, are regulated by crucial growth factors or cellular interactions, we are testing various polypeptide growth factors and extracellular matrix proteins for their effects on OE neurogenesis *in vitro*. We have also generated immortalized OE cell lines by using retroviruses to transduce oncogenes into cultured OE cells. One such cell line (derived from a primary OE basal cell culture) develops branching processes when transplanted into neonatal mouse brain—a condition in which cells from freshly isolated OE can undergo apparent morphological differentiation into neurons.

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In order to understand how neurons in the vertebrate nervous system are generated, whether during embryonic development or during regeneration,

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it is important to identify and to analyse functionally the molecules that regulate neurogenesis. To achieve this it is advantageous to establish an experimental system with which the process of neurogenesis can be directly examined and easily manipulated. Our approach to this problem has been to study neurogenesis in tissue culture. For these studies, we have used the olfactory epithelium (OE) of the mouse.

Several features of the OE make it well suited for *in vitro* studies of neurogenesis. Like the neural tube—the primordium of the vertebrate nervous system—the OE is an epithelial structure in which neurons are generated. However, the OE has a much simpler structure than the neural tube; in its mature form the OE contains only three or four cell types, and only a single type of neuron—the olfactory receptor neuron. In addition, the OE continues to generate neurons throughout life (Graziadei & Monti Graziadei 1978). Finally, because the OE maintains its capacity for neurogenesis, it has been possible for investigators to perform studies that show that neurogenesis in the OE is subject to regulation, presumably by cellular interactions (e.g. Graziadei 1973, Monti Graziadei & Graziadei 1979, Costanzo & Graziadei 1983, Camara & Harding 1984).

We have developed a tissue culture system for the olfactory epithelium and have demonstrated that olfactory receptor neurons are generated in these cultures. By identifying molecular markers for the different cell types present in the OE *in vivo* and *in vitro*, and by combining use of these markers with [<sup>3</sup>H]thymidine uptake analysis in quantitative studies of neurogenesis, we have begun to characterize the cellular stages in the neurogenic pathway (Calof & Chikaraishi 1988a,b, Calof & Chikaraishi 1989). Our current studies are focused on two goals: (1) to determine the role that cellular interactions play in regulating the proliferation of OE neuronal precursors and the differentiation of these precursors into neurons, both by analysing the effects of identified polypeptide growth factors and extracellular matrix molecules on OE cultures, and by transplanting OE cells back into various regions of the developing nervous system; and (2) to generate immortalized cell lines from OE cultures by retrovirus-mediated gene transfer, in order to facilitate biochemical and molecular genetic experiments on clonal populations of OE cells.

#### Cellular stages in olfactory neurogenesis

The conditions we use for culturing embryonic mouse OE have been described elsewhere (Calof & Chikaraishi 1989). Briefly, the nasal turbinate regions of Day 14 or 15 embryos are dissected, and the sensory epithelium is purified away from the underlying stroma by enzymic digestion and mechanical trituration. Pieces of purified OE are then explanted into culture in serum-free, defined medium, onto glass or plastic substrata that have been coated with various extracellular matrix molecules. Three types of cells appear in cultures prepared

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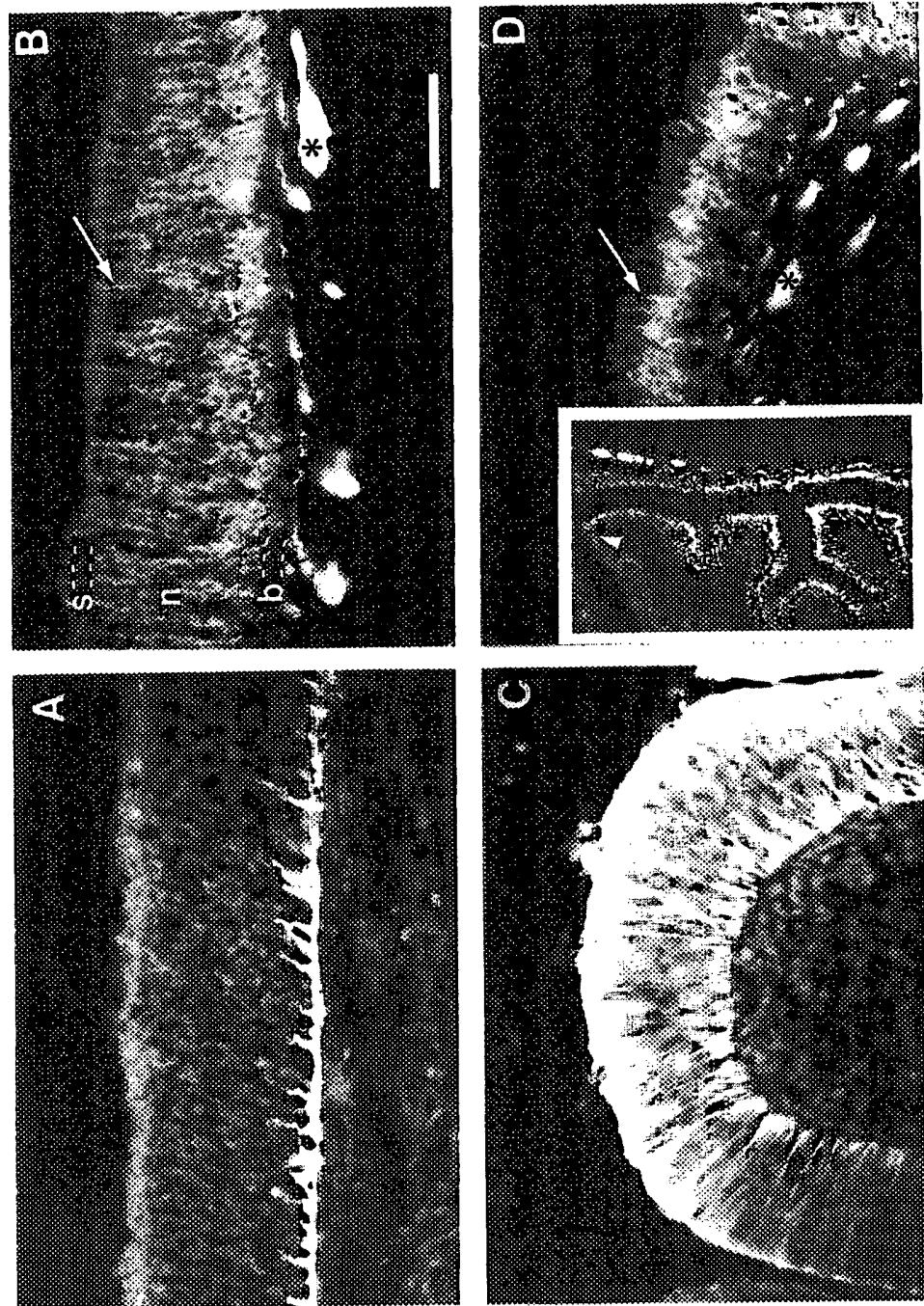
in this way: epithelial cells, which grow in flat sheets and are the *in vitro* equivalents of the basal cells; cells with phase-bright bodies and neurites, which are immature olfactory receptor neurons; and, at early times (< 24 hours) in culture, small round cells, which we now know to be the precursors of olfactory receptor neurons (see below).

Our ability to perform quantitative *in vitro* studies of olfactory neurogenesis has been aided by the development of molecular markers for these different cell types. Two markers in particular, antibodies that distinguish the basal cells and the receptor neurons of the OE, have proved particularly useful. The basal cells of the OE are distinguished by a commercially available antiserum to keratins (Calof & Chikaraishi 1989). The olfactory receptor neurons are stained by a monoclonal antibody that recognizes a cytoplasmic epitope common to the 140 and 180 kDa forms of the neural cell adhesion molecule, N-CAM (Calof & Chikaraishi 1989, DiFiglia et al 1989). In OE cultures, the antiserum to keratins exclusively stains the sheets of flat epithelial cells, indicating that these are the *in vitro* equivalents of the basal cells, and the monoclonal anti-N-CAM exclusively stains the neurite-bearing cells, which are the olfactory receptor neurons (Fig. 1; cf. Calof & Chikaraishi 1989).

Previous studies of neuronal regeneration in the OE *in vivo* suggested that the basal cells are self-renewing stem cells that ultimately give rise to the neurons in this system (Harding et al 1977, Monti Graziadei & Graziadei 1979). However, by means of quantitative [<sup>3</sup>H]thymidine uptake experiments performed in combination with immunocytochemical analysis, we have demonstrated that the small round cells, which appear at early times in OE cultures and are both morphologically and antigenically distinct from either basal cells or olfactory receptor neurons (Calof & Chikaraishi 1989), We have named these cells the immediate neuronal precursors (INPs) of olfactory receptor neurons. Our current hypothesis is that INPs are the *in vitro* equivalents of the so-called 'globose' basal cells that have been described occasionally in anatomical studies of olfactory epithelium (e.g. Graziadei & Monti Graziadei 1979).

The molecular markers we have developed for OE cultures have also made it possible to characterize the nature of INP divisions. Pulse-chase [<sup>3</sup>H]thymidine uptake autoradiography, combined with anti-N-CAM immunocytochemical analysis of OE cultures, demonstrated that proliferating INPs do not express N-CAM, but that more than 80% of their progeny are postmitotic, N-CAM-positive cells. This indicates that the majority of INP divisions produce two, N-CAM-positive sister neurons (Calof & Chikaraishi 1989). Thus, most INPs do not appear to behave like stem cells *in vitro*, but appear to be unipotential cells that give rise only to neurons.

Our observations on olfactory neurogenesis *in vitro*, combined with the results of others' investigations of olfactory receptor neuron regeneration *in vivo*, suggest a sequence of events that is schematized in Fig. 2. In this scheme the



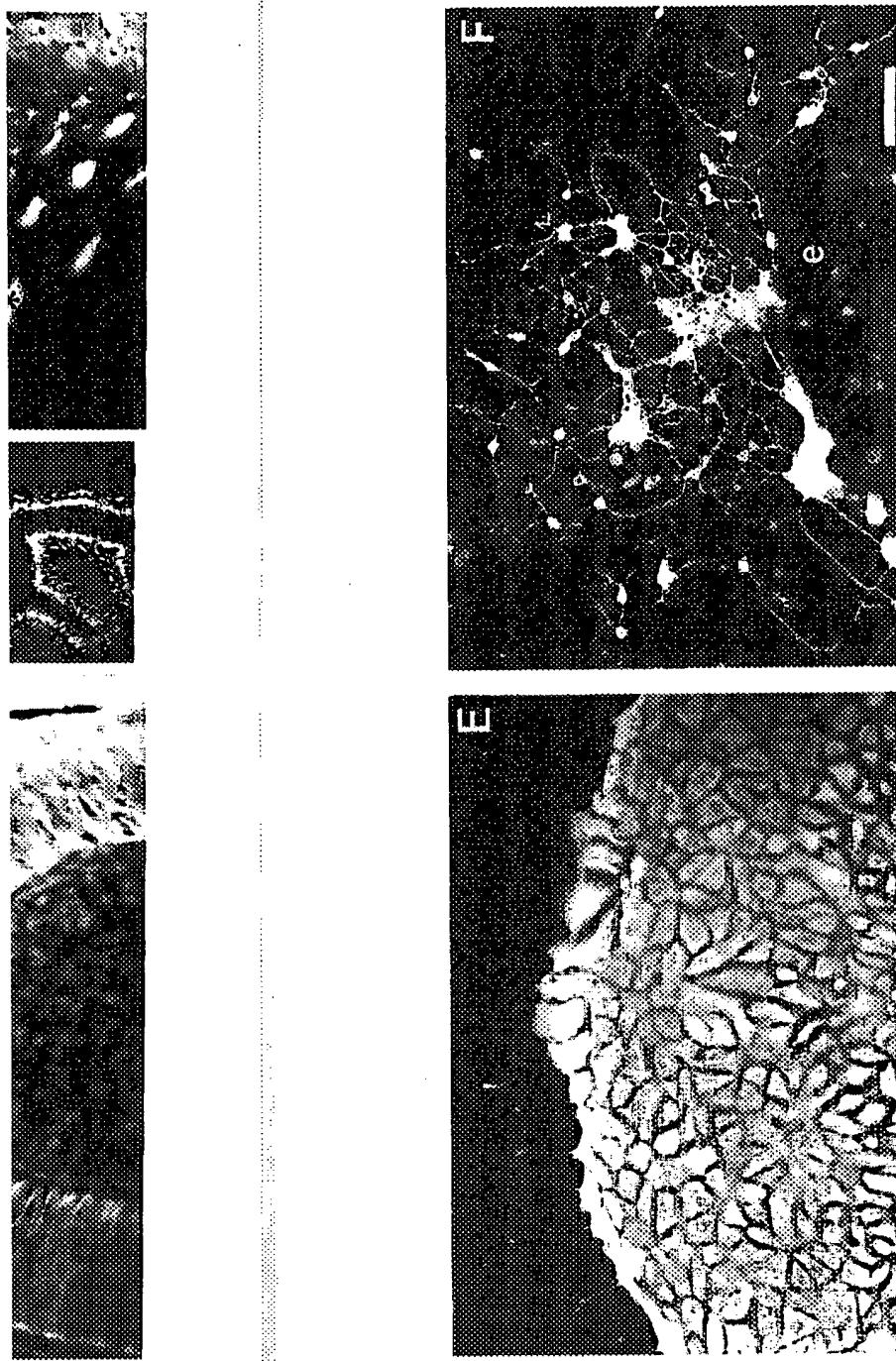
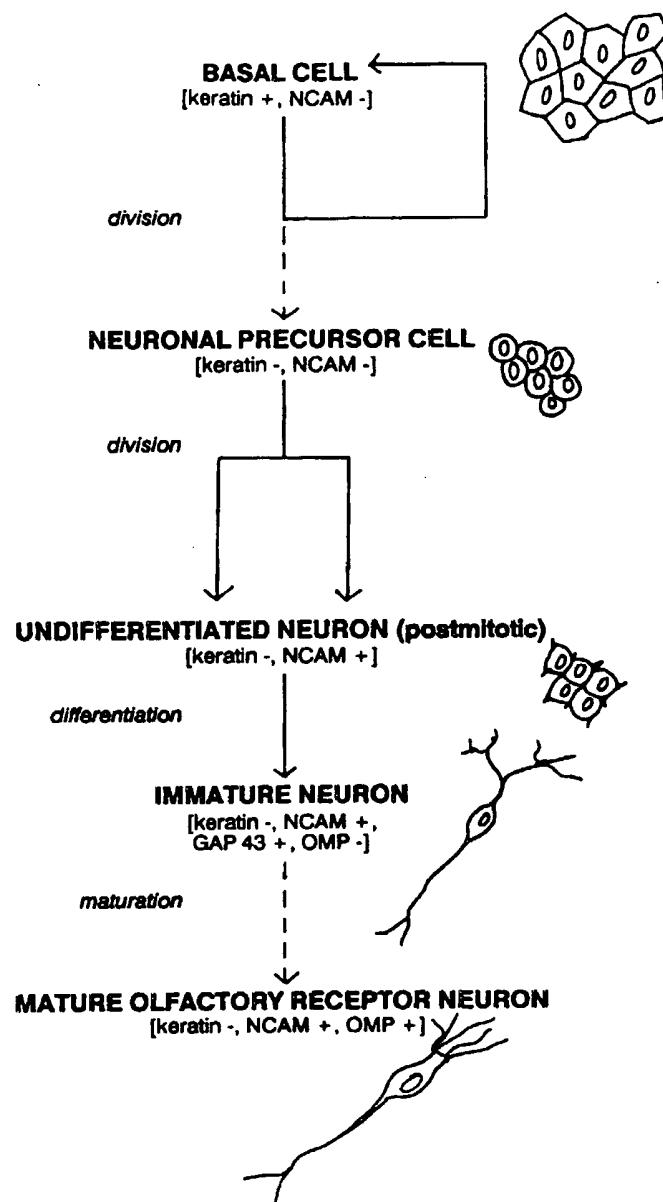


FIG. 1. Immunocytochemical distinction between mouse basal cells and olfactory receptor neurons *in vivo* and *in vitro*. A, C and E show staining with rabbit anti-keratins antiserum in postnatal mouse olfactory epithelium (OE), embryonic (E15) OE, and 36 h OE cultures, respectively. B, D and F show staining with monoclonal anti-N-CAM antibody in the same tissues. *In vivo*, anti-keratins antiserum exclusively stains the layer of basal cells immediately adjacent to the basement membrane in postnatal OE (A); in E15 OE (C), this antiserum stains about half of the cells, many of which extend from the basal to the apical surface. This staining pattern is consistent with the observation that, in OE at this age, the nuclei of proliferating cells are present throughout the basal-apical extent of the epithelium, and only later become restricted to the basal layer (Smart 1971). *In vitro*, the anti-keratins antiserum stains the sheets of cells with epithelial morphology (E). Monoclonal anti-N-CAM (B) exclusively stains neurons in the extensive receptor neuron layer (n), but does not stain cells in the basal cell layer (b) or the luminal sustentacular cell layer (s). Note that both the apical dendrites of olfactory receptor neurons (arrows, B, D) and the bundles of olfactory neuron axons (\*, B, D) are stained by the antibody to N-CAM. The anti-N-CAM antibody also clearly demarcates olfactory from nearby respiratory epithelium (arrowhead, D, inset). *In vitro*, monoclonal anti-N-CAM antibody stains the neurite-bearing cells (F). Bars, 50 µm. The bar in B also applies to A, C and D; the bar in F also applies to E. (Taken, with permission, from Calof & Chikaraishi 1989 © 1989 Cell Press.)



**FIG. 2.** Neurogenesis in the olfactory epithelium: a proposed sequence of events. The diagram shows a proposed series of steps in proliferation and differentiation leading to the production of olfactory receptor neurons via neuronal precursor cells (immediate neuronal precursors, INPs) (see text). Solid arrows indicate steps whose existence is directly supported by our observations. Dashed arrows indicate steps whose occurrence has been suggested previously by *in vivo* studies. (Taken, with permission, from Calof & Chikaraishi 1989 © 1989 Cell Press.)

solid lines represent events that we can confirm in tissue culture; the dashed lines are hypothesized steps based on suggestions made from *in vivo* studies. Our current experiments are directed towards answering three major questions that this scheme makes apparent:

1. Is the keratin-positive basal cell really a neuroepithelial stem cell? This question subsumes several further questions. Can we obtain evidence from *in vitro* experiments that the keratin-positive cell behaves like a stem cell—i.e. divides asymmetrically to regenerate itself and an INP? What is the stimulus for stem cell division and commitment to neuronal differentiation? Are there specific genes that direct neuronal determination?
2. Is it possible for an INP to undergo several rounds of division, analogous to the amplification divisions of committed progenitor cells in the haemopoietic system, before its progeny differentiate into neurons?
3. What are the conditions necessary for the long-term survival and biochemical maturation of olfactory receptor neurons?

#### Regulation of olfactory neurogenesis by intercellular interactions

We are using several approaches to gain some understanding of the way in which intercellular interactions might regulate neurogenesis in the olfactory epithelium. The first approach is to test the effects of polypeptide growth factors and conditioned media on the process of neurogenesis in OE cultures. If any of these agents affects OE neurogenesis *in vitro*, it is reasonable to assume that this should be detectable as (a) a change in the mitotic index of basal cells; (b) a change in the mitotic index of INPs; or (c) a change in the number of neurons generated in OE cultures.

Table 1 shows the results of experiments designed to determine whether various agents can sustain neurogenesis in OE cultures. Under normal conditions in OE cultures, the proliferation of INPs is ceasing by 24 hours; this is reflected in the low labelling index of INPs observed at this time in culture in pulse-fix [<sup>3</sup>H] thymidine uptake analyses (cf. Calof & Chikaraishi 1989, Fig. 3). In the experiments shown in Table 1, mouse OE cultures were grown in the continuous presence of the indicated agent. [<sup>3</sup>H] Thymidine was added after the first 24 hours of culture. After a further 24 hours in culture the labelling index of INPs + neurons was determined. In this type of experiment, an increase in the labelling index above control levels suggests that an overall increase in neurogenesis is being effected. However, it should be noted that such an increase could reflect any or all of the following biological events: an increase in basal cell-to-INP transitions; an increase in the percentage of INPs that proliferate; or a lengthening of the INP cell cycle.

Two effects are noteworthy (Table 1). The first is an apparent inhibition of neurogenesis by epidermal growth factor. EGF has a dramatic effect in promoting basal cell proliferation, but in long-term OE cultures it does not

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**TABLE 1 Effects of growth factors and conditioned media on neurogenesis in mouse olfactory epithelium cultures**

<i>Growth factor/conditioned medium (Concentration)</i>	<i>Labelling index (%) (SD)</i>
No growth factor or conditioned medium	6.5 (1.9)
Basic fibroblast growth factor (5 ng/ml) <sup>a</sup>	11.4 (1.3)
Acidic fibroblast growth factor (10 ng/ml) <sup>a</sup>	6.0 (0.6)
Transforming growth factor- $\beta$ (5 ng/ml) <sup>b</sup>	4.4 (1.0)
Epidermal growth factor (50 ng/ml) <sup>c</sup>	2.9 (0.4)
Nerve growth factor (2.5S; 50 ng/ml) <sup>d</sup>	4.4 (0.3)
BRL-CM (serum-free conditioned F12; 33%) <sup>e</sup>	6.5 (1.3)
STO-CM (serum-free conditioned F12; 33%) <sup>e</sup>	5.6 (1.2)

This test was designed to determine whether any of the added agents can promote the sustained generation of neurons in olfactory epithelium cultures. E15 mouse OE cultures were grown in serum-free, defined low-calcium medium as described in Calof & Chikaraishi (1989), except that carrier protein was increased to 5 mg/ml crystalline bovine serum albumin (ICN Biochemicals); this is the protein concentration that would be achieved in medium supplemented to approximately 8% calf serum. Cultures were grown on glass coverslips coated with laminin and fibronectin as described (Calof & Chikaraishi 1989) for 24 h in 1 ml of medium with growth factor or conditioned medium added at specified concentrations. After 24 h in culture the medium was removed and cultures were re-fed with 1 ml of fresh medium (again with growth factor or conditioned medium added) containing 0.1  $\mu$ Ci/ml [ $^3$ H]thymidine; 24 h later (total of 48 h in culture), cultures were fixed and processed for autoradiography as described in Calof & Chikaraishi (1989). Slides were exposed for eight days at -80°C, developed in D-19 developer, and stained with Hoechst 33258 (bisbenzamide, 1  $\mu$ g/ml). Labelled nuclei of INPs + neurons were counted using a 40  $\times$  phase objective. The labelling index is the percentage of INPs + neurons with silver grains over their nuclei. For each data point a minimum of two cultures was examined; for each culture, approximately 1000 cells in a minimum of 20 fields were scored.

<sup>a</sup>Basic and acidic FGF were purified from bovine brain by heparin affinity chromatography using published procedures (Lobb & Fett 1984).

<sup>b</sup>TGF- $\beta$ , purified from human platelets, was obtained from Collaborative Research, Inc. (Bedford, MA, USA).

<sup>c</sup>Recombinant human EGF was obtained from Amgen Biologicals (Thousand Oaks, CA, USA).

<sup>d</sup>2.5S NGF, purified from male mouse submaxillary gland, was obtained from Collaborative Research, Inc.

<sup>e</sup>Ham's F12, supplemented with defined culture additives (cf. Calof & Chikaraishi 1989) and glucose (0.3%), was conditioned for 48 h over confluent cultures of Buffalo Rat Liver cells or STO embryonic mouse fibroblasts (generous gifts of Dr Elizabeth Robertson, Dept of Genetics, Columbia University College of Physicians and Surgeons, New York, NY, USA).

appear to cause basal cells to generate INPs and thereby neurons (unpublished observations). It is possible that EGF promotes squamous differentiation of basal cells *in vitro*, thereby inhibiting basal cell-to-INP transitions and causing a net decrease in neurogenesis, as reflected in this assay. The other effect observed is a modest increase in labelling index caused by basic fibroblast growth factor. Here again, maintaining cultures for long periods of time in bFGF does not seem to trigger basal cells to generate neurons continuously, so the effect of bFGF on neurogenesis in this assay may be exerted directly

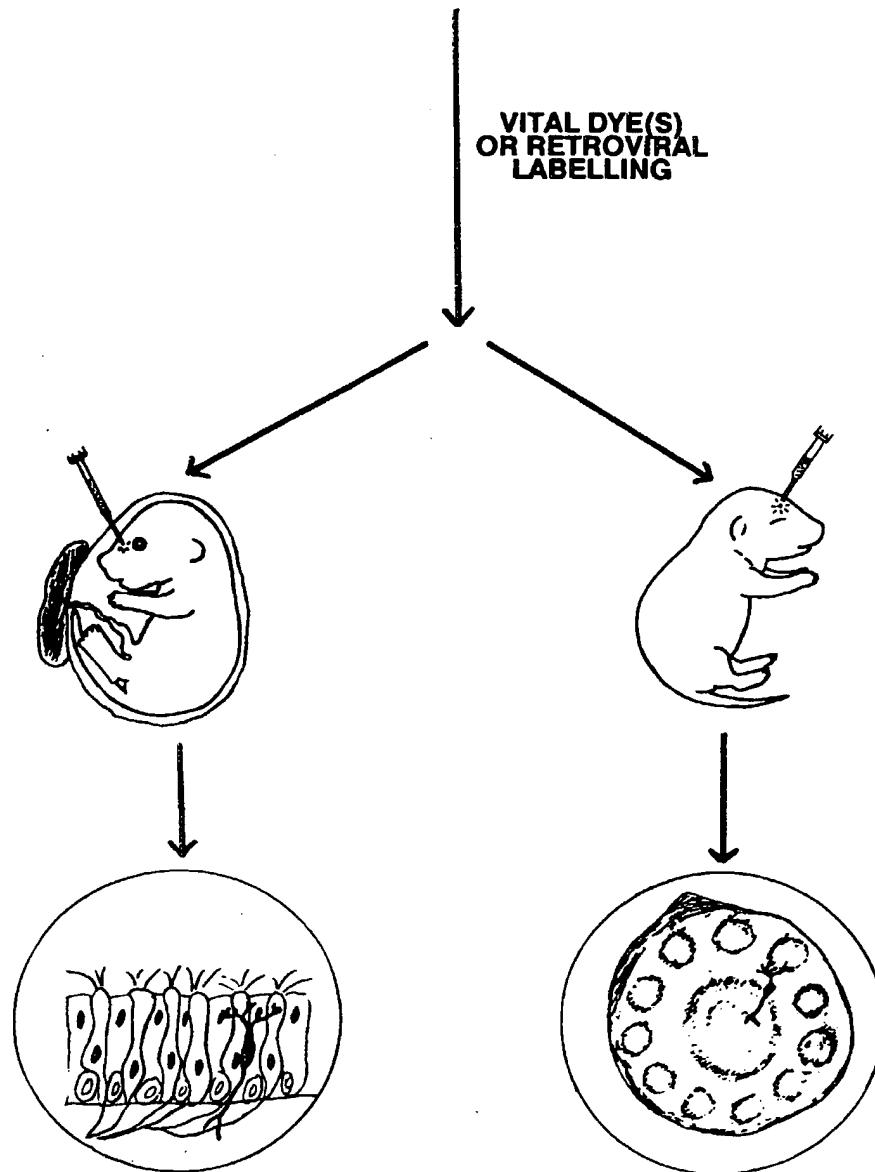
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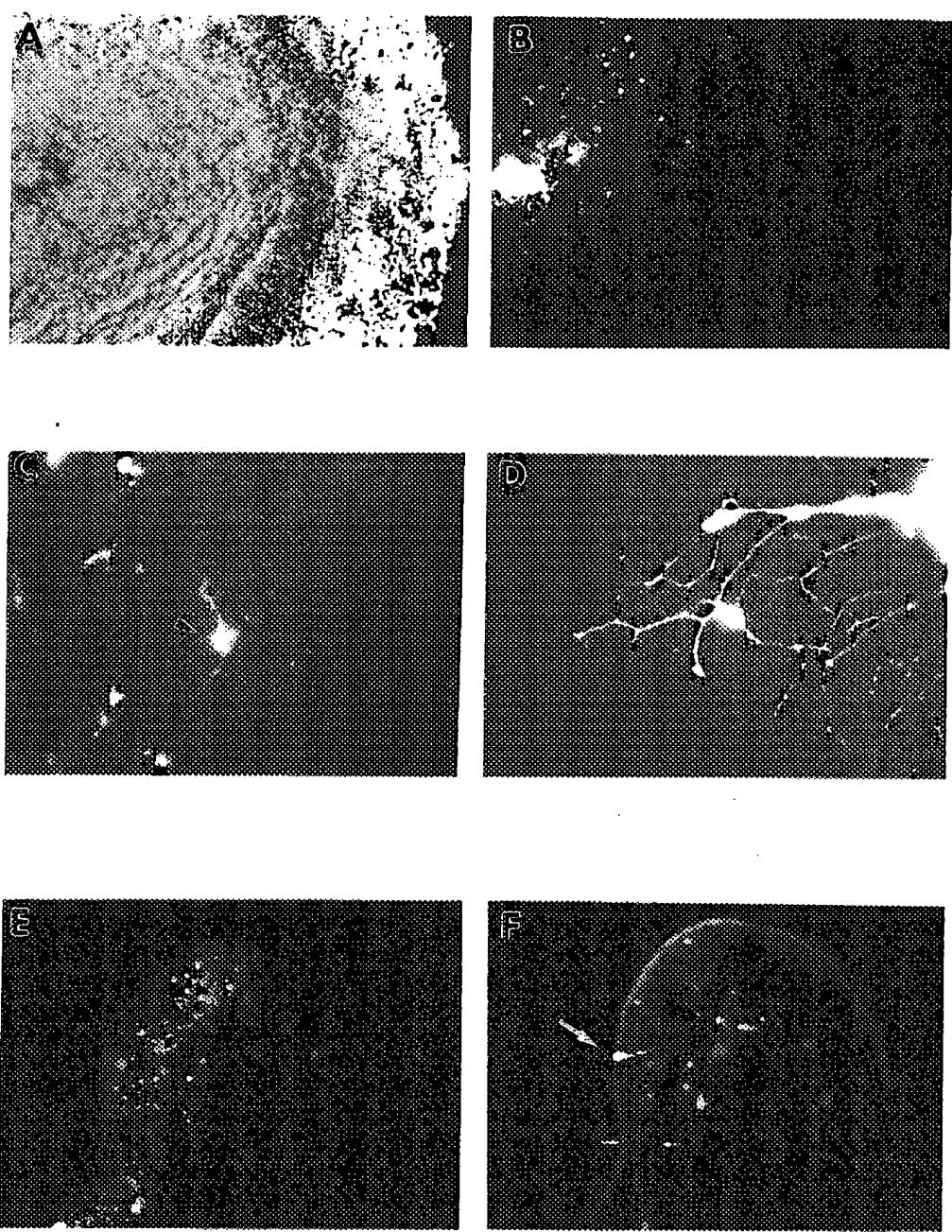
te the sustained growth in serum-supplemented medium (except that carrier proteins were omitted); this is the same medium as described by Hockings and colleagues (1986) containing calf serum and processed as described. Cells were plated onto eight-well chamber slides (Nunc) containing fibronectin-coated wells and processed for eight days at 33°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h, permeabilized with 0.1% Triton X-100 in PBS for 1 h, and processed for immunocytochemistry using rabbit anti-mouse nestin antibody (Developmental Studies Hybridoma Bank, Iowa City, USA) or mouse anti-β-tubulin antibody (Sigma). Cells were then processed for double immunostaining using biotin-conjugated goat anti-rabbit IgG and streptavidin-allophycocyanin (Biotium) and visualized using confocal microscopy (Leitz Labophot 2).

unpublished observations) and causing other effects (e.g. fibroblast proliferation) of time course continuously, treated directly

### PRIMARY OR IMMORTALIZED CELLS FROM OLFACTORY EPITHELIUM



**FIG. 3.** Transplantation of mouse olfactory epithelium cells. Primary or immortalized cells from olfactory epithelium are first labelled, either by means of a chemical dye, or by transducing a marker gene into them using a recombinant retrovirus. Labelled cells are then transplanted either into the olfactory epithelium of E14 host embryos or into the olfactory bulb of neonatal host mice. After five days the host animal is killed and its tissues are sectioned and examined for the presence of labelled cells with the characteristics of differentiated neurons.



on INPs (by, for example, promoting INP proliferation), rather than on basal cells. A more detailed experimental analysis of these effects is currently in progress.

So far, we have not succeeded in obtaining culture conditions for the olfactory epithelium in which neurogenesis continues for prolonged periods. In fact, in

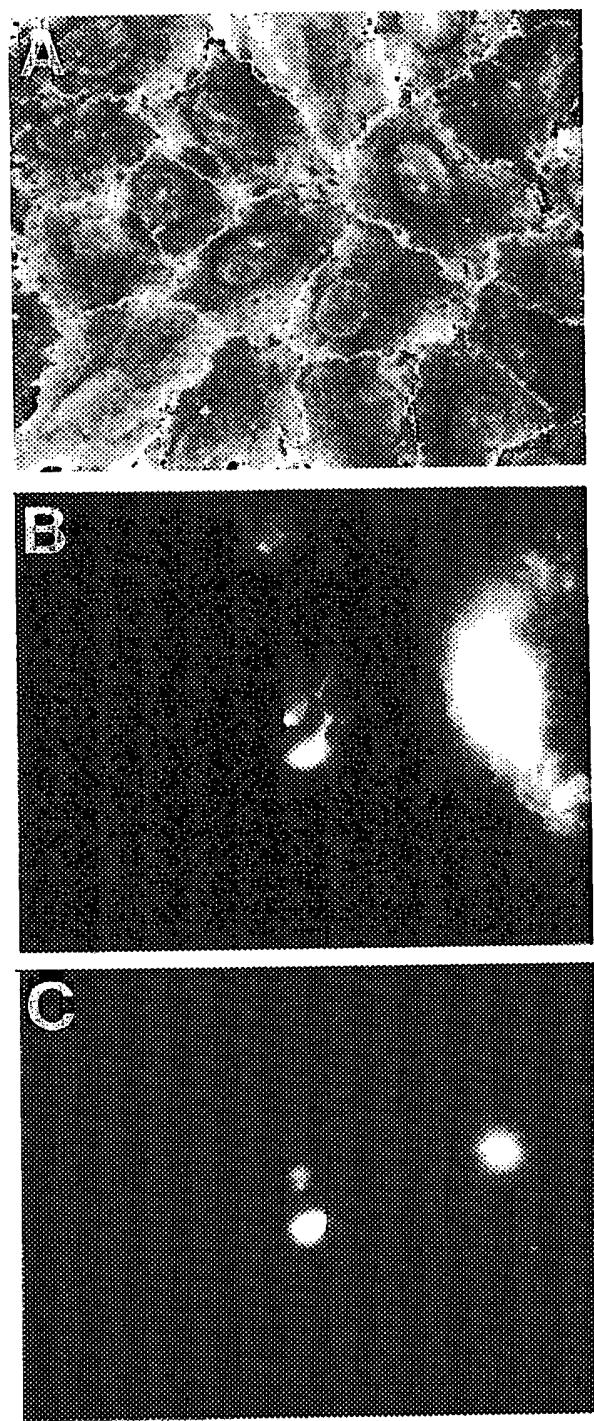
**FIG. 4.** Primary mouse olfactory epithelium cells can undergo neuronal differentiation after transplantation. Olfactory epithelium was purified from the turbinate regions of mouse embryos as described in Calof & Chikaraishi (1989). Tissue was labelled as described below and then transplanted into either the olfactory bulb of a neonatal host mouse (A-C) or into the olfactory epithelium of an embryonic (E14) host mouse (E,F) using *exo utero* surgical procedures (Muneoka et al 1986). Five days later the host animals were killed and host tissue was fixed in 4% paraformaldehyde in phosphate buffer and then sectioned on a vibratome (A-C) or cryostat (E,F).

**A-D:** Pieces of purified OE were incubated for 1 h at 37 °C in 40 µg/ml Dil (Honig & Hume 1986) in serum-free, defined medium ('normal-calcium medium'; cf. Calof & Chikaraishi 1989). The tissue was washed 10 times in a large excess of Hank's buffered salt solution (HBSS) containing antibiotics, 5 mM Hepes (pH 7.4), and fetal bovine serum (10%; first eight washes) or crystalline bovine serum albumin (BSA) (0.5 mg/ml; last two washes). Tissue was held in the dark on ice until transplanted by pressure injection through glass micropipettes. At the end of the surgical procedure the remaining tissue was placed into culture in low-calcium medium as described previously (Calof & Chikaraishi 1989).

A. Low power view of the host olfactory bulb stained with Hoechst 33258 (bisbenzamide, 1 µg/ml) to visualize cell nuclei. B. Low power view of Dil-labelled graft, same section. C. High power view of Dil-labelled cells in the transplant, same section. Note cell with bipolar neuronal morphology (arrowhead). D. Dil-labelled olfactory receptor neuron in cultures of the same tissue used for the graft shown in A-C. Note the morphological similarity between the transplanted cell in C (bipolar, with one thicker, more dendritic branching process, and one thinner branching process), and the cultured neuron in D.

**E,F:** Purified OE was spun to pellet (5 min at 100 × g), resuspended in 70 µl of 10 mg/ml lysinated rhodamine dextran (LRD, 10 000 M<sub>w</sub>; Molecular Probes) in phosphate-buffered saline (cf. Bronner-Fraser & Fraser 1988), and transferred to the cuvette of a Baekon-2000 Macromolecular Transfer System (Baekon, Inc, Saratoga, CA, USA). After 'Baekonization' (8 kV; 2<sup>10</sup> pulses; 0.8 s burst; 9 cycles; OPT 160), 20 µl of 1 mg/ml DNase I were added to the cuvette and the tissue was transferred to a sterile centrifuge tube and resuspended in a total volume of 6 ml of Hank's buffered saline solution. The suspension was underlaid with 3 ml of 4% crystalline BSA in HBSS and centrifuged (5 min at 100 × g) and the supernatant was removed. Labelled tissue was mixed with 3 µl of fluorescent-green latex microspheres (Fluoresbrite; Polysciences) and pressure-injected through a glass micropipette into the olfactory epithelium of mouse embryos *in situ*.

E. Fluorescent latex microspheres incorporated throughout the basal-to-apical extent of the OE, plus the underlying stroma, in the region of the host olfactory epithelium that received the graft. F. LRD-labelled cells in the same section. There are a number of LRD-labelled cells in the OE and underlying stroma, some out of the plane of focus. Note one labelled cell (large white arrow) integrated into the apical region of the OE, which appears to be extending a process toward the basement membrane of the epithelium. The basal-apical extent of the OE in this section is indicated by the two small white arrows.



our standard culture conditions using serum-free defined medium, all cells but basal cells die after about a week in OE cultures. However, these basal cells, while continuing to proliferate, do not generate INPs and neurons (cf. Calof et al 1989). There are at least two possible explanations for these results. First, the basal cell-to-INP transition may be a critical step in differentiation, requiring very specific conditions that we cannot yet reproduce *in vitro*; or, second, the keratin-positive basal cells may not be the progenitors of INPs and therefore ultimately of olfactory receptor neurons. It is thus of some importance to determine whether basal cells can, in fact, generate INPs and thereby neurons, and we are continuing to search for different conditions that might permit prolonged OE neurogenesis *in vitro*. However, we are also pursuing an alternative approach for determining whether the keratin-positive basal cells in OE cultures can generate neurons, and what conditions might permit the basal cell-to-INP transition. In this approach we transplant these cells back into an appropriate tissue to see if they show neurogenic potential *in vivo*. The basic strategy for these experiments is depicted in Fig. 3.

Our initial transplantation studies have focused on determining whether purified OE cells, prior to any time in culture, can survive and differentiate when transplanted into either the olfactory bulb or the olfactory epithelium of a host animal. Figure 4 shows examples of transplants into both the olfactory bulb of a neonatal host mouse (A-C) and the olfactory epithelium of an embryonic (E14) host mouse (E,F). OE cells can be labelled either by incubation with the carbocyanine dye, DiI (dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) (A-D), or by 'Baekonization' (see legend) in the presence of lysinated rhodamine dextran (E,F). After these labelling procedures, OE cells will survive and undergo at

FIG. 5. Immortalized basal cells extend processes when transplanted into the olfactory bulb. The experiments used an immortalized OE cell line, OEinfE1A8VB9T8, derived from an OE basal cell culture.

A. Fluorescence micrograph of OEinfE1A8VB9T8 cells *in vitro*, stained with monoclonal antibody OKT8. Note the epithelial morphology of these cells, similar to the morphology of OE basal cells in primary culture (Fig. 1).

B,C. OEinfE1A8VB9T8 cells extend branching processes when transplanted into the olfactory bulb of a neonatal host mouse. Subconfluent cultures of cells were double-labelled with DiI and Fast Blue before transplantation. Cells were incubated for 1 h at 37 °C in 25 µg/ml DiI in complete medium (DME/F12 with 10% fetal bovine serum), then rinsed three times in complete medium and three times in calcium- and magnesium-free HBSS. Cells were then incubated for 15 min at room temperature in 17 µg/ml Fast Blue in CMF-HBSS (cf. McConnell 1988), rinsed four times, dissociated in trypsin-EDTA, spun through a gradient of 4% crystalline BSA in CMF-HBSS, and pressure-injected into the host olfactory bulb through a glass micropipette. Five days later the host tissue was fixed in 4% paraformaldehyde, sectioned on a vibratome and examined for double-labelled cells with neuronal morphologies. B shows two DiI-labelled cells, one of which is clearly extending a branching process. C shows that both of these cells are also labelled with Fast Blue, confirming that both cells are derived from grafted tissue.

least some degree of morphological differentiation into neurons, either *in vitro* (D) or *in vivo* (C,F).

These results now make it possible to contemplate experiments that should answer a critical question about neurogenesis in this system: can purified basal cells, when transplanted back into either of these two locations, generate olfactory receptor neurons? Although we do not yet have the answer to this question, the results of the experiments discussed below suggest that the answer might be yes.

#### Immortalized cell lines from olfactory epithelium

We have derived immortalized cell lines from primary mouse olfactory epithelium cultures as potential sources of material for biochemical and molecular biological experiments on neurogenesis. Such cell lines are generated by infecting cultures with recombinant murine retroviruses. The viruses contain the coding sequences for various oncogenes, linked to a dominant selectable marker (the *neo* gene, which encodes a phosphotransferase that confers resistance to the neomycin analogue G418). Olfactory epithelium cultures are infected with virus and then grown in the presence of G418. G418-resistant cells are then passaged, cloned and characterized.

Figure 5 shows the results of experiments with a clonal OE cell line, which was derived by infecting an OE basal cell culture with a retrovirus encoding an adenovirus E1A12S oncogene (Cone et al 1988, Calof et al 1989). *In vitro*, these cells have an epithelial morphology, like that of basal cells in primary OE cultures (Fig. 5A). After transplantation into the olfactory bulb of a neonatal mouse, these cells (or their progeny), extend branching processes (Fig. 5B). These results suggest that these immortalized cells may be undergoing some degree of neuronal differentiation after transplantation. At present, however, we have evidence only for this morphological change; in order to determine whether these cells have undergone neuronal differentiation at the biochemical level it will be necessary to demonstrate that they express neuronal markers. To facilitate such studies, we are currently testing various marker genes, which we can use to detect transplanted cells, and, using double-label immunocytochemical analyses, assess the expression of neuronal markers by these cells as well. For example, OEinfE1A8VB9T8 cells have been made to express a foreign marker gene, encoding the human lymphocyte glycoprotein, CD8 (Fig. 5A) (Littman et al 1985). The CD8 cDNA was transduced into these cells with the retroviral vector MV7-T8 (Maddon et al 1986). Expression of human CD8 (=T8) can be detected immunocytochemically using the monoclonal antibody OKT8 (American Type Culture Collection Catalogue) in tissue culture or in tissue sections (Calof & Jessell 1986, Trevino et al 1990). MV7-T8 can infect mouse neuronal precursor cells and human CD8 is expressed by the neuronal progeny of these infected cells, whereas human CD8 is not normally expressed in any

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mouse tissues (Calof & Jessell 1986, Trevino et al 1990). This marker gene should be useful in transplantation experiments in which double-label immunocytochemical techniques are required to assess the biochemical state of differentiation of transplanted cells.

If these immortalized OE cells do prove to generate neurons *in vivo*, this result would be of significance for two reasons. First, it would suggest that the basal cells that are observed in primary OE cultures are indeed neuroepithelial stem cells; and, second, it would indicate that OE cells can still retain their neurogenic capacity after oncogene-induced immortalization. Cell lines such as these could be useful in several ways. They could be manipulated pharmacologically, to allow the identification of molecules such as growth factors, growth factor receptors, and intracellular signalling molecules that may be important in triggering or sustaining neurogenesis. In addition, such cell lines may be useful for the isolation, molecular cloning, and functional testing of genes that act as switches, controlling the cell-type transitions that occur between neuroepithelial stem cells and neurons (e.g. Davis et al 1987).

### Summary

The olfactory epithelium provides a unique system for studying cellular and molecular events in neurogenesis. *In vitro* studies of this tissue have shown that neurogenesis is a multistage process, involving a committed immediate neuronal precursor which divides to generate two daughter neurons. *In vivo* studies suggest that this precursor may be the progeny of a neuroepithelial stem cell that retains its neurogenic potential throughout life *in situ*. Finding the conditions that allow this transition from stem cell to committed precursor to occur *in vitro* will provide information about the kinds of factors that regulate neurogenesis; such factors may prove to have similar roles in all embryonic neuroepithelia, and will be candidates for agents that regulate regeneration of sensory epithelia in adult animals as well. Immortalized neural stem cells, derived from olfactory epithelium, may be useful sources from which to isolate genes that are important in directing the differentiation of neurons from their precursors.

### Acknowledgements

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## DISCUSSION

**Presson:** I thought there was a possibility that these small round cells, and the cells that they turn into, might be migrating LHRH (luteinizing hormone releasing hormone) cells?

**Calof:** Two separate groups (at the NIH and also at Rockefeller University) have shown that LHRH neurons of the hypothalamus are derived from the olfactory placode; early in development they migrate out of the placode, via the terminal nerve (which is another derivative of that placode) and migrate into the brain, to form the LHRH neurons (Wray et al 1989, Schwanzel-Fukuda & Pfaff 1989). That's one reason why I am interested in the migratory properties of these cells. The question as to whether or not the small, round cells—which we call the immediate neuronal precursors—or their neuronal progeny express LHRH *in vitro*, or whether all olfactory cells that migrate express LHRH, even *in vivo*, is still open. I have the marker that Dr Schwanzel-Fukuda used and I hope to do that experiment. I suspect that the immediate neuronal precursors (INPs) *in vitro* will not all be LHRH positive; I don't think it's a requisite for migration.

**Presson:** But isn't there a distinct possibility that these small round cells are not olfactory neuronal precursors at all, but are the LHRH precursors?

**Calof:** I don't think so; all the evidence we have suggests that they give rise to olfactory receptor neurons. I am more interested in the possibility of something like a transdifferentiation effect, and whether, for example, getting out of the olfactory epithelium and on to a particular pathway induces LHRH expression. There's evidence from a Japanese group that the hypothalamic environment is important for full-blown LHRH expression (Daikoku-Ishido et al 1990).

**Farbman:** The differentiation of the LHRH-positive cells comes from the medial part of the placode, and you are looking at embryonic cells of the turbinete regions, which derive from cells in the lateral part of the placode, so they are unlikely to be LHRH positive.

**Calof:** It would be interesting if LHRH was a determined characteristic of cells of that medial part of the placode, or whether any of these neuronal

precursors that had a migratory pathway open to them might be capable of expressing LHRH, and might be 'plastic', essentially.

*Farbman:* It could be!

*Reh:* Your antiserum to keratins stains basal cells and your monoclonal to N-CAM seems to stain everything else *in vivo* except the support cells; but the round cells in culture are not stained by either. So what is their equivalent *in vivo*?

*Calof:* These round cells have been referred to by several names, including the 'globose basal cell' by Graziadei & Monti Graziadei (1979), and the 'light basal cell'—the term that Mackay-Sim uses. In the latter study (see Breipohl et al 1986), as has also been confirmed by Schwartz et al (1989), the cells in the regenerating olfactory epithelium that take up [ $^3\text{H}$ ]thymidine after a pulse injection are not the keratin-positive 'dark' basal cells; they are the cells immediately above them that are referred to as the 'light' or 'globose' basal cells, or what I call the immediate neuronal precursors (INPs). So the cell type is there, but it is hard to see in the adult olfactory epithelium; whereas, at about E15, this epithelium is growing fast and therefore the numbers of round cells (INPs) are greater. I suspect that in the regenerating epithelium, what I call the 'round cell' or INP is the globose or light basal cell.

*Reh:* Does it undergo further cytokinesis, after it becomes one of those cells?

*Calof:* It divides once, to generate two neurons, as far as we can tell.

*Reh:* And does it make a single division *in vivo*, as *in vitro*?

*Calof:* Nobody knows. To show this you would need to be able to mark a single globose cell *in vivo*, and then look much later at the olfactory receptor neurons in the same animal, to see if there were more than two olfactory receptor neurons with the label.

*Burd:* Majorie Schwartz and John Kauer (1990) have evidence in support of a single division. In salamanders, they injected single globose basal cells (which you call immediate neuronal precursor cells) with rhodamine dextrans. If they wait long enough, that single cell becomes two neurons.

*Reh:* Dr Calof is looking for a multipotent neuroepithelial cell; I wonder if this might be a separate subpopulation that is undergoing only one round of cytokinesis?

*Calof:* I don't know! We have this cell that I call 'immediate neuronal precursor', which is probably analogous to a committed progenitor cell in the haemopoietic system. So is there really a *stem cell* in the olfactory epithelium that gives rise to the INP, and which cell is it? A subpopulation of the globose basal cells could be that stem cell population; or perhaps we are just not looking at the right conditions to allow the keratin-positive basal cells to act as stem cells *in vitro*. It's also possible that the keratin-positive basal cells are very slowly generating INPs all the time, and that the cellular interactions that up-regulate the division and renewal of the epithelium act only on the globose basal cell (i.e. the INP *in vivo*). Over the long term you would have a basal cell just blebbing out globose cells, which would be susceptible to whatever the stimulus

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is for regeneration. I think it's an open question. There's no evidence *in vitro* that the keratin-positive basal cell acts as a stem cell.

*Corwin:* In your explants, have you seen the globose cells producing neurons, *in vitro*? The staining should have shown it, if proliferation is going on in the tissue.

*Farbman:* You would have to section the explanted tissue to see this.

*Calof:* All our evidence indicates that the globose cell-equivalent (the INP) gives rise to neurons *in vitro*. To see whether the keratin-positive basal cells can give rise to INPs, I have looked at later cultures, which were essentially pure basal cells and where I hoped to see basal cell-to-INP transitions; but in no experiments of that type have I seen anything convincingly going through the entire proposed pathway. So I am trying to be conservative about it. It may be true; the keratin-positive basal cells may be the neural stem cells of the olfactory epithelium, but I have no convincing *in vitro* evidence of that.

*Raymond:* Where are the support cells, in your cultures?

*Calof:* Are there support cells in the olfactory epithelium of the mouse embryo at this stage (E15), and how would one find them? I see no evidence for support cells in these explants; the basal cell marker (anti-keratins) and the neuronal marker (anti-N-CAM) that we use don't recognize support cells in the adult, or, as far as I can tell, in the embryonic mouse olfactory epithelium (cf. Calof & Chikaraishi 1989). *In vivo*, at E15, cytokeratin-positive cells span the thickness of the epithelium (Fig. 1C of my paper). If we dissociate the epithelium into single cells and count the cells that express the basal cell marker and the neuronal marker, we find that about 60% of the cells are keratin positive and, of the remainder, three-quarters of them are N-CAM positive; one-quarter of the cells are negative for both markers. Those numbers fit with the behaviour of the round cells, the neurons, and the keratin-positive epithelial cells *in vitro* (cf. Calof & Chikaraishi 1989). I don't see sustentacular (support) cells as such. There is a marker for such cells, SUS-1, but it doesn't appear until around E20 in the rat. Is that the first time an organized support cell layer forms, Dr Farbman, or is this the time the antigen is first seen?

*Farbman:* The sustentacular cell layer is well established in the rat fetus on the 15th day of gestation, although they are not mature cells at this stage (Menco & Farbman 1985). At the 20th or 21st day, a few sustentacular cells are immunoreactive with SUS-1, a sustentacular cell-specific monoclonal antibody (Hempstead & Morgan 1983). The number of immunoreactive cells increases postnatally. We have been unable to correlate SUS-1 immunoreactivity with morphological changes seen with the scanning or transmission electron microscope.

*Calof:* So what is the evidence for an established sustentacular layer? I don't see anything in the embryo that looks like this cell layer, and in the adult our markers don't recognize sustentacular cells. It is possible that the sustentacular cells could form, *in vitro*, part of the keratin-positive population. But in the

adult that's certainly not true. So either they are dying in our cultures from E15 mouse embryos, or they are not there to begin with.

*Farbman:* They may not be dying in culture; they may remain in the body of your explant, and not migrate away from it as neuronal precursor cells do. You would probably be able to visualize the supporting cells in the main body of the explant by sectioning it and examining the sections with a microscope.

*Raymond:* Are you suggesting that there are two separate lineages, one for sustentacular cells and one for neurons?

*Farbman:* Yes.

*Calof:* Most of the data seem to indicate that, both in the regenerating olfactory epithelium and in development.

*Farbman:* When [<sup>3</sup>H]thymidine is injected into an animal, one rarely sees uptake in olfactory sustentacular cells. In the relatively rare instances when labelled sustentacular cells are seen they are usually in pairs, suggesting that the cells divide *in situ*.

There is an interesting side issue alluded to by Dr Costanzo (p 237), namely that reconstitution of olfactory epithelium occurs differently depending on the type of injury. If axotomy is done, reconstitution occurs by an increase in the number of cell divisions in the basal cell population and then differentiation of these cells into neurons. However, if the entire epithelium is destroyed with zinc sulphate, something very different occurs, because the basal cell progenitor cells are destroyed by this procedure. Reconstitution of the epithelium presumably occurs by division of epithelial cells from Bowman's glands or their ducts, but the epithelium reconstitutes as a respiratory epithelium, not an olfactory epithelium. Later, the olfactory epithelium gradually reappears (Smith 1951).

*Corwin:* Does the re-epithelialization nucleate around the Bowman's glands?

*Farbman:* I don't think this has been followed in strict detail. I was thinking of this when Dr Costanzo mentioned the boundary between olfactory and respiratory patches; the suggestion was that this was a re-epithelializing patch over an injury site. But there is complete loss of the basal layer after zinc sulphate treatment, and the so-called stem cells come from somewhere else—presumably from the Bowman's glands or their duct cells.

*Burd:* Within a couple of days the whole epithelium is covered by these cuboidal cells.

*Calof:* I thought that after zinc sulphate lavage in adult mouse, Dr Matulionis saw some sustentacular cell migration out of Bowman's glands. In support of that view, I believe the SUS-1 antigen is also present in Bowman's gland cells?

*Farbman:* Yes. The SUS-1 mAb stains the cytoplasm of almost all the supporting cells of the olfactory epithelium and Bowman's gland cells.

*Watt:* Dr Calof, were your E1A-infected basal cells keratin positive? I gather that when you transplant them, they produce neuronal processes?

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*Calof:* They were keratin positive when first immortalized; after many passages and recloning, the cells of this clone are not keratin positive. I don't know whether that's the notorious fickleness of intermediate filament expression in transformed cells, or whether the cell is actually not a basal cell; it has epithelial morphology, but perhaps is more like an immediate neuronal precursor (INP) that has been immortalized.

*Watt:* But basal cells that are not immortalized divide in culture?

*Calof:* Yes; they produce more keratin-positive basal cells in culture.

*Watt:* Have you transplanted them and seen neuronal differentiation?

*Calof:* Yes; we did this experiment once, and we thought we saw Olfactory Marker Protein expression by the transplanted cells. But this needs to be repeated, and it needs to be followed longitudinally as well.

*Watt:* So it might be better to stick with the uninfected basal cells?

*Calof:* If one wants to determine the developmental potential of the keratin-positive basal cell, yes. The reason for making cell lines is to be able to use them as sources of material for biochemical purification. One thing that is interesting about the E1A-immortalized basal cell line is that if it is really undergoing neuronal differentiation after transplantation, this suggests that the basal cell we observe *in vitro* may really be a neuroepithelial stem cell that can be immortalized and still retain neurogenic potential.

*Watt:* Does the cell line differentiate on a dish, as well?

*Calof:* No. It acts just like a primary basal cell in that respect, dividing to give rise to more of the same cells, but never generating immediate neuronal precursors (INPs) and then neurons.

*Burd:* My laboratory is studying the role of thyroid hormone in the development of the olfactory system. We have examined the influence of early administration of thyroid hormone and the effects of blocking thyroid hormone synthesis on the number of cells dividing in the olfactory epithelium and the number of mature olfactory receptor cells at different stages of development in the African clawed frog, *Xenopus laevis*. These results on the developing olfactory epithelium may be relevant to adult plasticity, since the olfactory epithelium can be replaced in adulthood and since thyroid hormone levels influence chemosensory functions in adult vertebrates. For example, McConnell and colleagues (1975) showed that hypothyroid patients have olfactory deficits that are reversed with the administration of thyroid hormones. In addition, Mackay-Sim & Beard (1987) presented evidence suggesting that thyroid hormones may be necessary for normal maturation of the olfactory receptor cells in adult mice. Therefore, while the following experiments are focused on neural development, they are likely to be applicable to adult plasticity as well.

Development in *Xenopus* tadpoles can be divided into three major periods: embryonic development (stages 1 to 35), larval development (stages 35 to 58), and metamorphic climax (stages 58 to 66). In addition, stages 49 to 54 are part of the premetamorphic period and stages 54 to 58 are part of the

prometamorphic period. These stages of development were defined by Nieuwkoop & Faber (1956) and are based on external and internal body characteristics.

There are two forms of thyroid hormone, thyroxine ( $T_4$ ) and 3,5,3'-triiodothyronine ( $T_3$ ). Thyroxine predominates as the circulating hormone, but is converted to  $T_3$  in peripheral tissues;  $T_3$  is the active form of the hormone. Amphibian metamorphosis is dependent upon thyroid hormone; without it, larvae grow but do not undergo metamorphosis. Plasma levels of thyroid hormones are very low in *Xenopus* prior to stage 54, begin to rise after stage 54, peak at stage 62, and decline slowly in later stages of metamorphosis (Leloup & Buscaglia 1977).

In all vertebrates, many aspects of neural development depend on thyroid hormone. A study of normal development of the olfactory nerve showed that the number of olfactory axons in the olfactory nerve of *Xenopus* tadpoles increased more rapidly after stage 54, the stage when thyroid hormone secretion begins (Burd 1991). We wondered to what extent development of the olfactory epithelium in *Xenopus* required or was influenced by thyroid hormone. To examine this, we implanted small pellets of 10% thyroxine in cholesterol and oil unilaterally, adjacent to the nasal capsule (hyperthyroid treatment), or grew larvae in 0.01% propylthiouracil (PTU), a drug that blocks synthesis of thyroid hormone (hypothyroid treatment). In both experiments, the treatment started at stage 48, well before the normal rise in thyroid hormone at stage 54.

Seven days after thyroxine pellets were implanted, larvae were injected with [ $^3\text{H}$ ]thymidine, to label dividing cells; three days later they were killed for autoradiography and electron microscopy. We observed that within ten days, the precocious thyroxine treatment stimulated a dramatic increase (two-fold) in the number of olfactory receptor cell axons, and hence an increase in the number of mature olfactory receptor cells (Burd 1988, 1990). In addition, from the autoradiography results we determined that there were at least 12 times the number of newly generated cells in the olfactory epithelium of hormone-treated larvae (Burd 1990). These results indicate that thyroxine can stimulate a striking increase in cell division and neuronal maturation in the olfactory epithelium of animals, before the normal rise in hormone levels.

We also examined the effect of thyroid hormone on normal development of the olfactory epithelium by studying hypothyroid larvae. Animals were placed in PTU at stage 48 and were killed along with age-matched siblings when the siblings reached stage 52 (before thyroid hormone levels rise) or when the siblings reached stage 58 (when thyroid hormone levels are high and rising). Some PTU-treated animals were also killed long after siblings had gone through metamorphosis. Quantitative analysis of the number of olfactory receptor cell axons revealed that the PTU treatment had no effect on axon number by comparison with stage 50 larvae, when both control and PTU-treated animals have very low levels of thyroid hormone. The number of olfactory axons in

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PTU-treated animals, however, was only half the number in stage 58 siblings, suggesting that the hypothyroid condition either prevented receptor cell maturation or blocked neurogenesis by acting on cell division of stem or precursor cells (Burd 1988). Paternostro & Meisami (1989) also find that hypothyroidism affects the development of the rat olfactory epithelium.

To determine whether hypothyroid conditions influenced cell genesis in the olfactory epithelium, we raised animals in PTU beginning at stage 48 and injected them and age-matched siblings with [<sup>3</sup>H]thymidine when the siblings reached stage 50 or stage 58. After one day of survival, the animals were killed and processed for autoradiography and light microscopy. While there was no difference in the thickness of the epithelium or in the number of labelled cells in the epithelium of PTU-treated animals compared with their age-matched, stage 50, control siblings, these parameters were statistically different between PTU-treated animals and the age-matched, control animals at stage 58. The olfactory epithelium was thinner and there were five times fewer [<sup>3</sup>H]thymidine-labelled cells in the hypothyroid larvae and significantly fewer labelled cells than in the younger PTU-treated animals (Burd & Thomas 1990). It is possible that at some point in larval development the olfactory epithelium requires thyroid hormones for cell genesis. These findings demonstrate that hypothyroidism during larval development affects cell genesis in the olfactory epithelium. This may account for the reduced number of receptor cells. Thyroid hormones may also be necessary for neuronal maturation, but we have not yet directly addressed this question.

Mackay-Sim & Beard (1987) showed that euthyroid hormone levels are necessary for normal maturation of the olfactory receptor cells in adult mice. We found that thyroid hormone levels may play a role in cell genesis in the developing olfactory epithelium and can accelerate neuronal maturation. I would like to suggest that thyroid hormone levels may also be important for receptor cell turnover in other sensory systems, and recommend that you keep this in mind as you design your future experiments.

*Rubel:* Your suggestion is very good; your study, and Anne Calof's, remind me however of something that those of us who were studying nervous system development many years ago went through, when most of the models came from regeneration. We discovered that regenerating systems didn't necessarily follow the same rules as developing systems. Here we are interested in the reverse; we are interested in regeneration. In both these cases we need to find out if the same kinds of rules apply during regeneration as during initial development. I don't think we have evidence in either of these experimental paradigms.

*Margolis:* In my lab we have tried to address this question in the mammalian olfactory system by studying the relative temporal sequence of expression of B50/GAP43 and the Olfactory Marker Protein (OMP), both in normal development and during regeneration in response to olfactory lesions. We showed (Verhaagen et al 1989, Biffo et al 1990) that during normal ontogeny,

B50/GAP43 is expressed in immature but not mature olfactory neurons, while the profile of OMP expression is quite the reciprocal of this and is expressed only in mature olfactory neurons. In the regeneration studies (Verhaagen et al 1990) this sequence of events is fully replicated for B50/GAP43 and OMP proteins and mRNAs, so long as the target olfactory bulb is present. However, if the olfactory bulb is extirpated, then the sequence is arrested and only immature B50/GAP43-positive neurons are seen. This model illustrates that with appropriate molecular probes regeneration can be shown to recapitulate, at least partially, the sequence of events seen during ontogeny. However, that is not to say that one is a complete, exchangeable and unambiguous model of the other.

*Costanzo:* I would like to ask Gail a hypothetical question. We have learned from experience in the clinics we have set up for patients with taste and smell disorders, resulting from, for example, polyps or nasal sinus disease, that there is in fact very little we can do to improve these senses. Corticosteroids help in some patients, but this is a short-term solution. In the case of head injury, we just have to wait and hope for improvement during recovery. Do you think that giving thyroid hormone might work?

*Burd:* In fact, as shown by McConnell and colleagues (1975), hypothyroid patients have olfactory deficits that can be reversed within about 14 days by administering thyroid hormone. So if the reason your patients can't smell is because they are hypothyroid, it is probably a good idea to try giving them thyroid hormone. However, if they have undergone some kind of trauma and the loss is due to an inability of the axons to contact the olfactory bulb, then thyroid hormone would probably not be beneficial.

*Costanzo:* These are not all trauma cases. There are many nasal diseases that involve presumably cellular mechanisms.

*Lewis:* Cancer patients who receive chemotherapy with cytotoxic drugs have unpleasant disorders of taste and smell. I am unaware of studies of how far this is a consequence of damage to the proliferating cells of the olfactory epithelium. Cytotoxic drug treatment for cancer is extremely common, and a large number of people are seriously affected by this. Distortions of taste and smell may contribute to nausea and loss of appetite.

*Oakley:* One view of that is conditioned aversion; people who get sick after eating will associate the malaise with food, and therefore are put off eating.

*Lewis:* That is the explanation given in the textbooks, but they don't seem to consider the possibility of damage to olfactory or taste epithelium and resultant disturbance of the sensory nerve connections.

*Oakley:* That would seem to be an additional factor. Besides conditioned aversion, there could be damage to the chemical senses.

In relation to hormonal effects on sensory receptors, years ago Allara (1952) and subsequently Zalewski (1969) found that testosterone treatment of rats caused a few taste buds to form on the tongue at sites where they were normally

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apparent only during development. It seems as though some quiescent stem cells were reactivated in adults. This observation has not been followed up.

*Fernald:* I would certainly agree that thyroxine would be an interesting hormone to study. The September 1990 issue of the *Journal of Neurobiology* is devoted to metamorphosis. A postdoctoral fellow in my lab, Dr Barbara Evans, has evidence that in flounder (*Pleuronectes americanus*) which undergo metamorphosis, thyroxine triggers rod production (Evans & Fernald 1990). Premetamorphic animals have a retina with one, cone-like kind of photoreceptor. At metamorphosis, concomitant with the appearance of T<sub>4</sub> and probably triggered by it, is the production of rods. A wave of neurogenesis is thus associated with thyroxine.

*Rubel:* Do you know if that's a direct triggering of the stem cells?

*Fernald:* Almost! A Japanese group showed that thyroxine, put into the eye of a flounder, causes it to migrate before it would normally have done so (i.e., before metamorphosis) (Inui & Miwa 1985). We are doing similar experiments and it looks as if thyroxine is one of the major triggers for the remodelling of the retina that occurs at metamorphosis. That issue of the *Journal of Neurobiology* contains articles on a range of metamorphic phenomena that occur in the natural life of the animal.

*Reh:* On the question of functional recovery in the olfactory system, to what extent is the appropriateness of the connections necessary for recovery of function?

*Costanzo:* There is a good topographical projection of the olfactory epithelium onto the olfactory bulb. One hypothesis suggests that olfaction is encoded by a spatial pattern of activity. An experiment that we hope to do is to selectively block axon projections from specific areas of the epithelium, preventing them from projecting back to the olfactory bulb, after regeneration. We could then test to see if hamsters are capable of discriminating odours. These hamsters would be compared to those where the projections are allowed to go back to their normal targets. My guess is that any unique projection pattern that can be discriminated from other patterns can be used to identify different odours. For example, you may have one pattern for the odour produced by an orange and another for that of an apple. After regeneration the 'orange' pattern may change to an 'apple' pattern in the brain, but if you keep associating it with an orange, you may relearn that that's an orange. In other words, unique patterns can be used to code sensory information but the patterns don't have to be the same every time in every individual.

*Ryals:* Using HRP retrograde labelling of olfactory neurons, could you follow endings back to their original position? In other words, is there a map of the periphery in the olfactory bulb?

*Costanzo:* So far, it looks as if axons go to the closest and most convenient spot. In order to see if they go back to selected targets, one would need to block the target area and see if they go around the block and go back to their target,

or if they go around it and then get diverted. Those experiments have not been done yet.

*Rubel:* It's my understanding that there is a beautiful topographical relationship between the epithelium and the bulb. However, the patterning mechanism for olfactory stimuli is quite controversial and whether the unique topography is important for the patterning mechanism is still unresolved.

*Costanzo:* Some people believe there are selective projections and others believe there are diffuse projections; some believe that both exist. My belief is that it is a mixture, and there are some selective areas that are more focused than others and there is a distribution of information across a population of cells. Either way, you still get a unique pattern; regardless of how the information is projected from the periphery, you can produce a unique cortical pattern that can be associated with a unique sensation.

*Oakley:* It is also fair to say that there's not uniform agreement that there is any significant topographical representation in the olfactory system. John Kauer (1991) and I feel that anatomical topographical projection is marginal. In rainbow trout, David Riddle and I (unpublished work) have found no evidence for topographical projections. We made focal injections of rhodamine and fluorescein-conjugated beads in the glomerular layer of the olfactory bulb, where they were retrogradely transported to the mucosa. You cannot tell from the mucosal labelling pattern whether the bead injection sites were widely separated or close together in the trout olfactory bulb.

*Palay:* But it could be different in different species?

*Oakley:* There are certainly species differences. In the rat, some very careful work shows a degree of regional topographical representation of receptor axons (Astic et al 1987).

*Costanzo:* In the frog *Rana catesbeiana*, we recorded from second-order cells in the olfactory bulb, showed that by stimulating different positions along the nasal epithelium, some cells will only be excited by a single area, whereas adjacent cells could receive input from all areas of that epithelium (Costanzo & Mozell 1976). Thus, there may be a mixture of topographical and diffuse projection onto cells in the bulb.

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